Inactivation of liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase by *o*-phthalaldehyde

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The two activities of chicken liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase were inactivated by *o*-phthalaldehyde. Absorbance and fluorescence spectra of the modified enzyme were consistent with the formation of an isoindole derivative (1 mol/mol of enzyme subunit). The inactivation of 6-phosphofructo-2-kinase by *o*-phthalaldehyde was faster than the inactivation of fructose-2,6-bisphosphatase, which was concomitant with the increase in fluorescence. The substrates of 6-phosphofructo-2-kinase did not protect the kinase against inactivation, whereas fructose-2,6-bisphosphate fully protected against *o*-phthalaldehyde-induced inactivation of the bisphosphatase. Addition of dithiothreitol prevented both the increase in fluorescence and the inactivation of fructose-2,6-bisphosphatase, but not that of 6-phosphofructo-2-kinase. It is proposed that *o*-phthalaldehyde forms two different inhibitory adducts: a non-fluorescent adduct in the kinase domain and a fluorescent isoindole derivative in the bisphosphatase domain. A lysine and a cysteine residue could be involved in fructose-2,6-bisphosphate binding in the bisphosphatase domain of the protein.

INTRODUCTION

Liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) is a bifunctional enzyme which catalyses the synthesis and breakdown of fructose 2,6-bisphosphate (Fru-2,6- P_2) [1,2]. The protein is composed of two identical M_r -55000 subunits [3]. Limited proteolysis and treatment of the enzyme with protein-modifying reagents led to the conclusion that the PFK-2 and FBPase-2 reactions are catalysed at separate sites on each subunit [4,5]. The primary structure of the protein has been determined by sequencing [6] and by analysis of a full-length cDNA [7]. Several domains have been identified. The PFK-2 domain contains four cysteine residues, at positions 107, 160, 183 and 198 [6], which are thought to be involved in maintaining the conformation of the sugar phosphate binding site [8]. In addition, two lysine residues may form part of this site [9]. The ATP-binding site has not been labelled or allocated yet. However, two consensus sequences for nucleotide-binding sites containing lysine have been identified (residues 48–54 and residues 274–281) [6]. The FBPase-2 domain, which is in the C-terminal half of the protein, contains the active-centre histidine at position 258, and shares sequence and functional homology with both yeast phosphoglycerate mutase and human bisphosphoglycerate mutase [10]. The N-terminal domain contains serine-32, which is phosphorylated by cyclic AMP-dependent protein kinase [6].

o-Phthalaldehyde has been used as an active-site inhibitor of succinic semialdehyde dehydrogenase [11], aldolase [12], cyclic AMP-dependent protein kinase [13], fructose-1,6-bisphosphatase [14] and hexokinase [15]. The compound reacts with amino and thiol groups, forming an isoindole adduct, which exhibits strong fluorescence. Moreover, the wavelength of the emission

maximum gives information on the microenvironment of the modified residues [16]. In the present paper, we show that *o*-phthalaldehyde is a potent inhibitor of PFK-2 and FBPase-2 and that the mechanism of inhibition of the two activities is different.

MATERIALS AND METHODS

o-Phthalaldehyde was obtained from Janssen, Beerse, Belgium. On the day of the experiment, fresh solutions of the compound were prepared in methanol and diluted in distilled water. Chicken liver PFK-2/FBPase-2 was purified to homogeneity as described [17,18]. The enzyme preparations (1–2 mg/ml) were stored at -80°C in a buffer containing 20 mM-Hepes, 50 mM-KCl, 5 mM-MgCl₂, 1 mM-potassium phosphate, 0.1 mM-phenylmethanesulphonyl fluoride, 10% (v/v) glycerol, and 15 mM-2-mercaptoethanol, pH 7.5. The specific activity of three different preparations of PFK-2 was between 50 and 100 munits/mg of protein. All biochemicals were from Boehringer or Sigma. Radioactive iodoacetamide was purchased from Amersham International, Amersham, Bucks., U.K.

Incubation of PFK-2/FBPase-2 with *o*-phthalaldehyde and measurement of enzyme activities

PFK-2/FBPase-2 (20 $\mu\text{g/ml}$) was incubated at room temperature in a buffer containing 100 mM-sodium borate, 2 mM-EDTA, 5 mM-MgCl₂, 1 mM-potassium phosphate, pH 8.4 (borate buffer), with the indicated concentrations of *o*-phthalaldehyde. The reactions were started with enzyme, and samples were removed at various times and mixed with an equal volume of 20 mM-cysteine/5 mM-2-mercaptoethanol at 0°C to stop the reaction. Samples containing 0.5 μg of protein were

Abbreviations used: Fru-2,6- P_2 , fructose-2,6-bisphosphate; FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105).

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assayed for PFK-2 [19] and FBPase-2 [20], with concentrations of substrates and ligands as indicated in the legends to the Figures and Table.

Spectral studies

PFK-2/FBPase-2 (0.4 mg/ml) was incubated in 0.4 ml of borate buffer at room temperature with 50 μ M-*o*-phthalaldehyde. After 1 h of incubation, the reaction was stopped by adding 100 μ l of 20 mM-cysteine/5 mM-2-mercaptoethanol. The mixture was dialysed overnight at 4 °C against 1 litre of borate buffer containing 10% glycerol and 1 mM-dithiothreitol. Absorption spectra were recorded in 0.4 ml micro-cuvettes in a Perkin-Elmer Lambda 5 UV/Vis spectrophotometer. For fluorescence spectra, the enzyme was diluted to a protein concentration of 0.1 mg/ml, and the excitation and emission spectra were recorded at emission and excitation wavelengths of 430 nm and 337 nm respectively in a Perkin-Elmer LS 5 luminescence spectrometer. PFK-2/FBPase-2 protein concentration was determined by the method of Lowry *et al.* [21], with bovine serum albumin as a standard. The protein concentration of dialysed enzyme was calculated from the $A_{280} - A_{400}$ difference, knowing that a difference of 0.933 corresponds to a solution of PFK-2/FBPase-2 containing 1 mg of protein/ml.

For the measurement of fluorescence, PFK-2/FBPase-2 (20 μ g/ml) was incubated in borate buffer with the indicated concentrations of *o*-phthalaldehyde at room temperature. The reactions were started with enzyme, and the increase in fluorescence was monitored (excitation wavelength 335 nm; emission wavelength 455 nm). Pseudo-first-order rate constants for the increase in fluorescence were calculated by the Guggenheim method [22]. Measurements of fluorescence were also performed on PFK-2/FBPase-2 which had been pre-treated with iodoacetamide or *N*-ethylmaleimide. The enzyme (0.25 mg/ml) was first incubated with 5 mM-iodoacetamide or 5 mM-*N*-ethylmaleimide at room temperature. After 1 h, the reactions were stopped with 0.1 ml of 20 mM-cysteine/5 mM-2-mercaptoethanol, and the mixtures were dialysed overnight against 1 litre of borate buffer containing 10% glycerol and 0.1 mM-dithiothreitol. The preparations were then diluted to a protein concentration of 0.1 mg/ml and treated with 50 μ M-*o*-phthalaldehyde as described above.

Labelling of PFK-2/FBPase-2

The isoindole derivative of PFK-2/FBPase-2 (4 μ g) was prepared as described above and incubated with 10 μ M-[2- 32 P]Fru-2,6- P_2 as described [20]. To label the enzyme with [14 C]acetamide, PFK-2/FBPase-2 was treated with or without *o*-phthalaldehyde as described above for the spectral studies, and incubated with 1.5 mM-[1- 14 C]iodoacetamide (57 μ Ci/ μ mol) in 0.05 ml of borate buffer at 30 °C. Samples (5 μ l) were removed for the determination of protein-bound radioactivity [23].

Titration of total thiol groups with dithionitrobenzoate

The isoindole derivative of PFK-2/FBPase-2 (55 μ g) was prepared as described above. After 1 h, the enzyme was precipitated with an equal volume of 16.4% (w/v) trichloroacetic acid, and the mixture was left at 0 °C for 10 min. After centrifugation (5 min, Eppendorf Microfuge) the protein pellet was resuspended in 50 μ l of 0.5 M-NaOH by heating for 10 min at 80 °C. The denatured protein was incubated with 0.1 mM-dithionitrobenzoate

in a final volume of 1 ml of 100 mM-sodium borate/2 mM-EDTA, pH 8.4. The reaction was followed by monitoring the increase in A_{412} . The stoichiometry of thiol groups modified per mol of holoenzyme was calculated from the absorption coefficient (13.6 mM $^{-1}$ ·cm $^{-1}$) and a M_r value of 110000 for PFK-2/FBPase-2.

RESULTS

Inactivation of PFK-2 by *o*-phthalaldehyde

Incubation of liver PFK-2/FBPase-2 with *o*-phthalaldehyde led to the irreversible loss of PFK-2 activity. A semi-logarithmic plot of the time course of inactivation by different concentrations of *o*-phthalaldehyde showed non-linearity and tended towards curvature (Fig. 1). Assuming that the initial rate of inactivation was pseudo-first-order, we calculated the apparent first-order-rate constants (k_{app}) from plots of the negative logarithm of percentage residual activity versus time. The reciprocals of the initial rate constant were plotted against the reciprocals of *o*-phthalaldehyde concentration, and a linear relationship was obtained (Fig. 1 inset).

Fig. 1. Time course of inactivation of PFK-2 by *o*-phthalaldehyde

PFK-2/FBPase-2 was incubated as described in the Materials and methods section with the indicated concentrations of *o*-phthalaldehyde. At various times, the reaction was stopped by the addition of an equal volume of 20 mM-cysteine/5 mM-2-mercaptoethanol, and samples were removed for PFK-2 assay (room temperature, sub-optimal conditions: 50 μ M-fructose 6-phosphate and 500 μ M-MgATP); 100% of PFK-2 activity corresponds to 40 munits/mg of protein under these conditions. Rate constants (k_{app}) were calculated from plots of the negative natural logarithm of residual activity versus time; the initial rates corresponding to the first 40–60 s were taken. The reciprocal of k_{app} was plotted against the reciprocal of the concentration of *o*-phthalaldehyde (inset).

The data are consistent with the reversible formation of an enzyme-inhibitor complex (E·I) before irreversible enzyme modification (eqn. 1), as for affinity labelling [24]:

For this model, a linear relationship between the reciprocals of $k_{app.}$ and of inhibitor concentration has been described (eqn. 2), and corresponds to the inset of Fig. 1.

$$1/k_{app.} = (K_i/k_2)(1/[I]) + (1/k_2) \quad (2)$$

It is thus possible to estimate K_i [the concentration giving half-maximal inhibition, equal to $(k_{-1} + k_2)/k_{+1}$] for *o*-phthalaldehyde, and k_2 , the rate constant for the formation of the adduct. For three different enzyme preparations, K_i was $28 \pm 4 \mu\text{M}$ and k_2 ranged from 0.021 to 0.048, with an average value of $0.033 \pm 0.014 \text{ s}^{-1}$.

A study of the kinetic properties of PFK-2 which had been inactivated by 50% showed that *o*-phthalaldehyde decreased $V_{max.}$, but had no effect on the apparent K_m values for the substrates, MgATP and fructose 6-phosphate, nor on the sensitivity towards inhibition by *sn*-glycerol 3-phosphate.

Spectroscopic studies

Treatment of PFK-2/FBPase-2 with $50 \mu\text{M}$ -*o*-phthalaldehyde for 1 h completely inactivated PFK-2 and resulted in the formation of a derivative that displayed an absorbance peak at 337 nm, which is a characteristic feature of an isoindole derivative. However, formation of the isoindole derivative was not linked to the inactivation of PFK-2, but did result in FBPase-2 inactivation (see below and Fig. 4a). From the absorption coefficient of the isoindole derivative [25], it was calculated that 0.88 ± 0.04 (mean $\pm$ S.E.M. for three separate experiments) mol of isoindole/mol of PFK-2/FBPase-2 subunit was formed. Titration of the total number of thiol groups in denatured control and *o*-phthalaldehyde-treated enzyme by dithionitrobenzoate showed that 1.27 mol of thiol/mol of enzyme subunit was not accessible in the treated

enzyme. The modified PFK-2/FBPase-2 was fluorescent, with excitation and emission peaks at 338 and 428 nm respectively (Fig. 2), which are also characteristic of an isoindole.

The rate of isoindole derivative formation measured by the increase in fluorescence depended on the concentration of *o*-phthalaldehyde. As for PFK-2 inactivation, K_i and k_2 , the rate constant for adduct formation, could be calculated from the double-reciprocal plot of the apparent first-order rate constants for the increase in fluorescence versus inhibitor concentration. From two experiments performed with separate enzyme preparations, K_i was $27 \mu\text{M}$ and k_2 was 0.014 s^{-1} . The K_i value is not different from that obtained for PFK-2 inactivation, whereas k_2 is less than half the averaged value for PFK-2 inactivation.

Inactivation of FBPase-2 by *o*-phthalaldehyde

A comparison of the effect of *o*-phthalaldehyde on PFK-2 and FBPase-2 activities (Fig. 3) showed that, after 30 s, the concentration required to inhibit PFK-2 by 50% was about 10 times less than for FBPase-2. Comparison of the time course of FBPase-2 inactivation and fluorescence increase revealed (Fig. 4a) that the two phenomena were concomitant. By contrast, the inactivation of PFK-2 was faster. This confirms the measurements of different values of k_2 for PFK-2 inactivation and fluorescence increase. On the other hand, the addition of 5 mM-dithiothreitol after 30 s of incubation (Fig. 4b) partially arrested both the increase in fluorescence and FBPase-2 inactivation, whereas PFK-2 inactivation proceeded. This experiment clearly indicates that the inactivation of PFK-2 can be dissociated from that of FBPase-2. It suggests that the mechanism of inactivation is different, since the formation of an isoindole derivative was not linked to PFK-2 inactivation. It also suggests that *o*-phthalaldehyde forms an adduct which inactivates PFK-2, but which is different from isoindole, since it does not involve thiol groups.

PFK-2/FBPase-2 whose FBPase-2 activity had been

Fig. 2. Fluorescence spectra of PFK-2/FBPase-2 isoindole adduct

PFK-2/FBPase-2 isoindole adduct was prepared as described in the Materials and methods section. Excitation (a) and emission (b) spectra were recorded.

inactivated by 97% could not be phosphorylated from $[2\text{-}^{32}\text{P}]\text{Fru-2,6-}P_2$. In addition, a study of the kinetic properties of FBPase-2 which had been inactivated by 75% showed that *o*-phthalaldehyde decreased $V_{\max}$, but

Fig. 3. Dose-dependence curves for the inactivation of PFK-2 and FBPase-2 by *o*-phthalaldehyde

PFK-2/FBPase-2 was incubated with the indicated concentrations of *o*-phthalaldehyde as described in the Materials and methods section. After 30 s the reactions were stopped by the addition of an equal volume of 20 mM-cysteine/5 mM-2-mercaptoethanol, and samples were taken for the measurement of PFK-2 (●) and FBPase-2 (■). PFK-2 was assayed at room temperature (optimal conditions: 2 mM-fructose 6-phosphate and 5 mM-MgATP). FBPase-2 was assayed at room temperature in the presence of 2 μM - $[2\text{-}^{32}\text{P}]\text{Fru-2,6-}P_2$ (50 000 c.p.m./nmol), 1 mM-MgGTP, 1 mM-*sn*-glycerol 3-phosphate and 1 mM-potassium phosphate; 100% of PFK-2 and FBPase-2 activity correspond to 100 and 20 munits/mg of protein respectively.

had no effect on the apparent K_m for Fru-2,6- P_2 , nor on the sensitivity towards inhibition by fructose 6-phosphate (results not shown).

Protection by substrates and ligands

PFK-2/FBPase-2 was incubated with substrates and ligands to test for protection against *o*-phthalaldehyde-induced inactivation and fluorescence increase. The kinase substrates, MgATP and fructose 6-phosphate, tested alone or in combination, exerted little, if any (about 10%), protective effect on PFK-2 activity and fluorescence increase (results not shown). In contrast, there was a dose-dependent protection of FBPase-2 inactivation by its substrate Fru-2,6- P_2 , which at 20 μM concentration completely protected against inactivation

Table 1. Protection by Fru-2,6- P_2 against FBPase-2 inactivation and fluorescence increase induced by *o*-phthalaldehyde

PFK-2/FBPase-2 (20 μg) was incubated with and without 50 μM -*o*-phthalaldehyde in 0.5 ml of borate buffer as described in the Materials and methods section. For measurement of FBPase-2 activity, the reaction was stopped after 4 min with 0.5 ml of 20 mM-cysteine/5 mM-2-mercaptoethanol, and samples (5 μg of protein) were assayed for 5 min in the presence of 5 μM - $[2\text{-}^{32}\text{P}]\text{Fru-2,6-}P_2$, 1 mM-potassium phosphate, 1 mM-MgGTP and 1 mM-*sn*-glycerol 3-phosphate. For fluorescence measurement, the reaction was stopped with 5 mM-dithiothreitol and measurements were made as described in the Materials and methods section.

Fru-2,6- P_2 added	FBPase-2 (% of initial activity)	Fluorescence (% of total)
None	37	100
3 μM	55	—
10 μM	70	—
20 μM	100	63

Fig. 4. Time course of fluorescence increase and of inactivation of PFK-2/FBPase-2 induced by *o*-phthalaldehyde

(a) PFK-2/FBPase-2 was incubated with 50 μM -*o*-phthalaldehyde as described in the Materials and methods section. At the indicated times, samples were removed and PFK-2 (○) and FBPase-2 (△) activities were assayed as described in the legends to Figs. 1 and 3 respectively. In a parallel experiment, fluorescence was monitored. The relative fluorescence (□) was calculated at the indicated times. (b) The experiment was carried out as described above, except that 5 mM-dithiothreitol was added after 30 s of incubation; 100% of PFK-2 and FBPase-2 activity correspond to 19 and 4.5 munits/mg of protein respectively.

(Table 1). However, 20 μM -Fru-2,6- P_2 only partially protected against the *o*-phthalaldehyde-induced increase in fluorescence.

PFK-2/FBPase-2 modification by iodoacetamide, *N*-ethylmaleimide and *o*-phthalaldehyde

Alkylation of rat liver PFK-2/FBPase-2 by iodoacetamide activates the kinase without affecting the bisphosphatase. It modifies cysteine residues at the hexose phosphate binding site in the kinase domain [8]. On the other hand, *N*-ethylmaleimide inhibits PFK-2 without affecting FBPase-2 activity; like iodoacetamide, it reacts with thiol groups involved in sugar phosphate interaction at the kinase site [8]. To test the possibility that *o*-phthalaldehyde reacts with the same cysteine residues as those modified by iodoacetamide and *N*-ethylmaleimide, PFK-2/FBPase-2 was treated with iodoacetamide or *N*-ethylmaleimide, dialysed, and then made to react with *o*-phthalaldehyde. The pre-treated enzymes could react with *o*-phthalaldehyde, as evidenced by the increase in fluorescence. By the same token, the isoindole derivative of PFK-2/FBPase-2 could react with iodoacetamide, as evidenced by the same incorporation of [^{14}C]acetamide into both the *o*-phthalaldehyde-treated and untreated enzymes. Therefore, and in agreement with the results described above, these experiments suggest that isoindole is formed by reaction with thiol groups that are not involved in sugar phosphate binding in the kinase domain.

DISCUSSION

Treatment of chicken liver PFK-2/FBPase-2 with *o*-phthalaldehyde inactivates both PFK-2 and FBPase-2. The inactivation of FBPase-2 probably results from the formation of a stable derivative whose absorbance and fluorescence properties are characteristic of isoindole. The stoichiometry of adduct formation is approx. 1 mol of isoindole formed/mol of enzyme subunit. The isoindole derivative is believed to be formed by a reaction between *o*-phthalaldehyde and amino and thiol groups in the protein. If, as is the case for rat liver PFK-2/FBPase-2 [6], the *N*-terminal amino acid of the chicken liver enzyme is *N*-acetylated, then *o*-phthalaldehyde cannot react with this residue and probably reacts with the ϵ -amino group of a lysine residue and an adjacent thiol group. The PFK-2 active site contains not only two lysine residues, but also four cysteine residues which are involved in maintaining the conformation of the sugar phosphate binding site, and which can be specifically labelled by iodoacetamide [6,8]. Our data indicate that these cysteines are not involved in the formation of the isoindole derivative. Indeed, PFK-2/FBPase-2 pre-treated with iodoacetamide can still react with *o*-phthalaldehyde, and the PFK-2 substrates exert very little protection against inactivation. By contrast, the protection against *o*-phthalaldehyde-induced FBPase-2 inactivation by Fru-2,6- P_2 suggests that the isoindole derivative is formed in the bisphosphatase domain. It also suggests that a lysine and/or a cysteine residue may be involved in binding Fru-2,6- P_2 to the phosphatase site near to histidine-258 in the folded protein.

The polarity of the microenvironment surrounding the isoindole derivative of PFK-2/FBPase-2 can be indirectly assessed by comparing the molar transition energy (E_T) of PFK-2/FBPase-2 derivative with that of a model

isoindole derivative in various solvents. The molar transition energies can be calculated from the fluorescence emission maximum of an isoindole by the empirical relationship [12,13]:

$$E_T = 2.985 \lambda_{em} - 1087.28 \quad (3)$$

where λ_{em} is the wavelength of the fluorescence emission maximum of the isoindole derivative. The calculated E_T for the PFK-2/FBPase-2 derivative (180 kJ/mol) is greater than those reported for the derivatives of aldolase (136 kJ/mol [12]), the catalytic subunit of protein kinase (121 kJ/mol [13]), fructose-1,6-bisphosphatase (121 kJ/mol [14]) and hexokinase (116 kJ/mol [15]), and is close to that of isoindole in acetonitrile or dioxane. Therefore, the microenvironment of the site modified by *o*-phthalaldehyde in PFK-2/FBPase-2 is less hydrophobic than in these other enzymes.

Two main arguments indicate that *o*-phthalaldehyde-induced inactivation of PFK-2 does not result from isoindole formation. These are: (i) the difference in the rate constants (k_2) for PFK-2 inactivation and for the increase in fluorescence, and (ii) the persistence of PFK-2 inactivation when the increase in fluorescence was abolished by dithiothreitol. Therefore, the irreversible inactivation of PFK-2 probably results from the formation of another adduct which is not fluorescent and does not involve thiol groups. Since PFK-2 substrates did not protect against inactivation, this adduct is probably located at a site which is different from the substrate-binding sites. The fact that *o*-phthalaldehyde can inactivate PFK-2 without forming an isoindole emphasizes the caution that should be taken when *o*-phthalaldehyde is used as a fluorescent probe for enzyme active sites.

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